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Simultaneous quantification of six major allergens in commercial foods for children using a Multiplex Array on a Digital Versatile Disc

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Abstract

The hypothesis of this study is centered around the logic that an enhanced analysis of potential allergens during the food production can lead to increased accuracy and reliability of food labeling. The development of a cost-effective and straightforward optoelectrical microanalytical system for the simultaneous quantification of the six most common food allergens (peanut, hazelnut, almond, milk, wheat, and soybean) is presented. The system uses a regular versatile disc (DVD) functionalized with highly selective antibodies in a microarray format and a DVD drive as the optical detector. The multiplexed assay reliably (RSD < 20%) determines the level of the allergenic proteins ranging from 0.1 to 143.4 ng mL^{-1}. The analysis of food consumables (biscuits, seafood substitutes, and probiotic foods) revealed a 100% accuracy in identifying the allergens ingredients declared on the label. The method offers potential for application as a high throughput biosensing tool for screening multiple allergens in commercial foods.

1. Introduction

Food protein allergy is an adverse immunologic response triggered by exposure to allergenic food proteins (Iweala et al., 2018; Sena-Torralba et al., 2020). There has been an increase in the worldwide prevalence (reported to be around 4% in children and 1% in adults) in the past two decades, setting off the alarms of what many consider to be a "second wave" of the allergy epidemic, following the "first wave" of asthma, allergic rhinitis, and inhalant sensitization (Elghoudi & Narchi, 2022). Interestingly, food allergies are more common in children than adults, responsible for a 33% increase in hospital admissions over the last 20 years in the UK (Rotella & Oriel, 2022).

Among the 200 foods proven as allergenic, the ones that account for the most severe disease are peanut, tree nuts, milk, wheat, soy, egg, fish, shellfish, and sesame (Elissa M. Abrams & Becker, 2015). Although there are some treatments aimed to mitigate the effects of allergic reactions, such as that for peanut allergy, which the FDA has recently approved (Palforzia, 2022), individuals who are allergic must simply avoid certain allergen suspicious foods (Jia & Evans, 2021). Avoidance, however, is becoming more and more complicated as almost 70% of children suffering from food allergies have adverse reactions to more than one type of allergenic food (Elissa Michele Abrams et al., 2020), which increases the risk of accidental exposure to allergens. Unintentional exposure to allergens has been reported to range from 14 to 50% (Quake et al., 2022). This fact is mainly due to 1) cross-contamination during the production process, 2) inappropriate labeling and 3) failure to read labels (Michelsen-Huisman et al., 2018; Sheth et al., 2010). As most food products are composed of multiple ingredients and nutrients, it is imperative to provide complete and accurate food labeling. Despite the European regulatory agency introducing the obligation to declare 14 allergenic ingredients on food labels (REGULATION (EU) No 1169/2011 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 October 2011, 2011), no regulatory threshold exists apart from that of gluten, which is set at 20 mg kg^{-1}(Sena-Torralba et al., 2020). In this sense, it is intuitive that reliable and accurate analytical methods are required with the capability to quantify multiple allergens simultaneously.

The most widely used protein-based methods for allergen detection are enzyme-linked immunosorbent assay (ELISA) and lateral flow assay (LFA). ELISA generally has a higher analytical sensitivity (µg L^{-1} range) than LFA (mg L^{-1} range), but the requirement of instrumentation and qualified personnel limits its use in laboratory settings. Conversely, the simplicity and portability of LFA promote its on-site application by non-specialized users (Parolo et al., 2020; Sena-Torralba et al., 2022). However, most commercial LFA kits rely on the individualized detection of food-borne allergens, since this usually allows for better analytical performance (*Fast and Reliable Test Kits for Food Allergen Detection*, 2022). Recently, a multiplexed LFA has been developed for the qualitative determination (visual limit of detection LoD of 0.1 mg L^{-1}) of casein, ovalbumin, and hazelnut allergenic proteins in commercial biscuits (Anfossi et al., 2019).

Moreover, the two analytical methods which have proven high suitability for multiplexing purposes are mass spectrometry (Henrottin et al., 2019; Sun New et al., 2018) and nucleic acid-based techniques (Suh et al., 2019, 2020). These methods have enabled the simultaneous detection of up to 13 and 10 food-borne allergens and allergen-coding genes in highly processed foods, respectively (Cheng et al., 2016; Ogura et al., 2019). Additionally, these approaches serve as exciting alternatives to using immunoassays to identify allergens in processed foods. Processed foods have undergone mechanical and chemical operations that promote the unfolding and aggregation of the proteins, which hinders their binding capacity to IgE and IgG antibodies (Clare Mills et al., 2009). To this end, mass spectrometry relies on the identification of the amino acid sequences of the proteins and thus is structurally independent (Monaci et al., 2018). Besides, the nucleic acid-based methods aim at detecting the allergenic protein-coding genes, which are far more stable than the proper proteins. However, despite their high accuracy and reproducibility, these approaches have shown low sensitivity (LoD of 3.9 mg Kg^{-1} of the target peptides and relative LoD of 50 mg kg^{-1} of target proteins, respectively), are complex, and usually require unportable equipment, limiting their applicability for on-site detection.

The integration of microarray technology to microchip-based biosensors has provided high-throughput and multiplexing capabilities. For instance, a giant magnetoresistive biosensor has been developed for the simultaneous detection of peanut and gliadin allergens achieving one order of magnitude higher sensitivity than ELISA (< 10 µg L^{-1}) (Ng et al., 2016). Similarly, an interferometric biosensor has shown to be capable of simultaneously detecting six food allergens in 6.5 minutes (LoD > 100 µg L^{-1}), with the possibility of re-use at least ten times (Angelopoulou et al., 2018). However, these signal transduction strategies are challenging to interpret via a non-specialized end-user and are incompatible with a direct visual inspection of the assay result. In contrast, xMAP technology, which is based on color-coded fluorescent magnetic beads as detectors, has proven high sensitivity (LoD 5 µg L^{-1}) and accuracy when detecting 15 food allergens simultaneously. It enables a fast qualitative evaluation of the generated signal. Still, quantification is restricted to the use of the commercial reader (Bio-Plex 200), and the analysis of 36 samples through assay requires around 6 hours (including sample pre-treatment) (Cho et al., 2015).

Herein, the study presents a simple, cost-effective, and portable biosensing approach to simultaneously quantify six food-borne allergens that cause the most severe disease afflictions in children (peanut, hazelnut, almond, milk, gliadin, and soybean). The biosensing platform consists of a standard digital compact disc that has been functionalized with capture antibodies in microarray format, enabling the high-throughput evaluation of 20 samples in 70 minutes **(Figure 1 A)**. The sensing method relies on the precipitation of TMB catalyzed by the HRP-conjugated detector antibodies. The blue colorimetric displayed on each microarray spot depends on the concentration

of the target analyte. It can be observed qualitatively by the naked eye (for a quick assessment) **(Figure 1 B),** or it can otherwise be accurately quantified with a portable modified DVD-drive and homemade hardware. The investigation has already validated this biosensing system for assessing immunoglobulin E (IgE) sensitization to allergens, leading to excellent results in terms of sensitivity and specificity (Juárez et al., 2021; Mas et al., 2020). In this study, we optimized key operation parameters with the aim of (1) avoiding non-specific signals upon the integration of the six assays in a single microarray, (2) combining non-competitive and competitive assay formats, and (3) including a robust positive and negative control assay, which improves the reliability of the results.

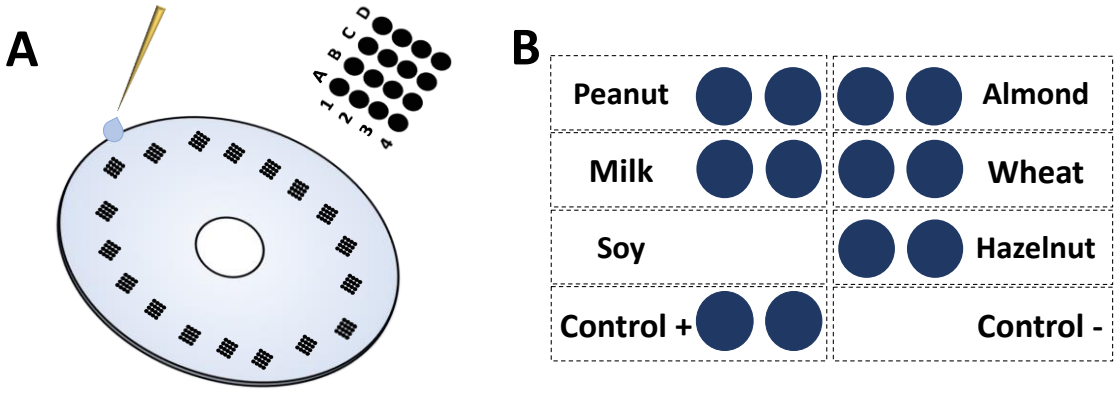

Figure 1. Schematic representation of the (A) DVD functionalized with the capture bioreceptors in microarray format (20 arrays, 4 rows, and 4 columns). (B) Microarray layout for detecting peanut, almond, milk (β-lactoglobulin), wheat (gliadin), and hazelnut in non-competitive assay format and soy (trypsin inhibitor) in competitive assay format. The microarray includes a positive and negative control assay.

2. Materials and methods

2.1. Reagents, materials, and instruments

Monoclonal anti-gliadin AMR5 capture antibody (M.30.GLU.IR5), HRP-conjugated anti-gliadin detection antibody (M.30.GLU.J01205n), gliadin standard solution (M.30.GLU.A04210c), polyclonal anti-β-lactoglobulin capture antibody (M.30.BLG.IPOLIC) and HRP-conjugated anti-β-lactoglobulin detection antibody (M.30.BLG.J01205n) were purchased from Ingenasa (Madrid, Spain). Trypsin inhibitor from Glycine max (soybean) (T6414), β-lactoglobulin from bovine milk (L-3908), bovine serum albumin (A7030), anti-Mouse IgG (Fab specific) antibody produced in goat (M6898) and polysorbate 20 (Tween 20) was supplied by Merck (Darmstadt, Germany). HRP Conjugation Kit - Lightning-Link® (ab102890) was purchased from Abcam (Cambridge, United Kingdom). Tetramethylbenzidine (ep(HS)TMB-mA) substrate was purchased from SDT GmbH (Baesweiler, Germany). Sodium phosphate buffer (PBS, 8 mM Na2HPO4, 2 mM, 137 mM NaCl, 2.7 mM KCl, pH 7.4), PBST (PBS with polysorbate 20 0.05% v/v), were prepared with purified water (Milli-Q, Millipore Iberica, Darmstadt,

Germany) and filtered through 0.2 µm polyethersulfone membranes (Merck, Darmstadt, Germany). CD Rohling-up GmbH (Saarbrücken, Germany) and LG Electronics Inc. (Englewood Cliffs, United States) supplied standard DVDs and DVD drive. A non-contact printing device (AD1500) was purchased from BioDot, Inc. (Irvine, United States). Hand blender (BA5607) was supplied by Solac S.A. (Vitoria-Gasteiz, Spain).

2.2. Extraction and purification of peanut, almond, and hazelnut proteins

Peanut, almond, hazelnut and soy proteins were, extracted at room temperature by adding 5 mL PBS (10 mM, pH 7.4) to 0.5 g of grinded product, followed by rotation for 1 hour. The mixture was centrifuged for 30 s at 3000 x *g* and the supernatant was filtered over a 0.45 µm filter. The protein content was determined by measuring the absorbance at 280 nm, and the Bradford method confirmed the data. The protein extracts were characterized by 15% SDS-PAGE gel electrophoresis (200 V for 40 min.).

2.3. Preparation, purification, and characterization of peanut, almond, hazelnut, and soy antibodies

Monoclonal antibodies (Mabs) were produced following an already published protocol (Yokoyama et al., 2013). In this case, the mice were immunized with 350 µg of the crude peanut, almond and hazelnut protein extracts and with the pure soybean trypsin inhibitor. Then the Mabs were isolated from raw cell culture media (approximately 1000 mL) by slowly adding 1000 mL of saturated ammonium sulphate solution under constant stirring. After standing still for 30 min, Mabs were collected by centrifugation for 10 min at 8000 x *g*. The obtained pellet was dissolved in PBS in a volume 1/10 of the original and dialysed for 48 h at 4°C. Then Mabs were further purified by affinity chromatography using HiTrap Protein G columns in accordance with the manufacturer's protocol. The Mab concentration was determined using a spectrophotometer, measuring the absorbance at 280 nm.

2.4. Microarray design and fabrication

The capture antibodies for the detection of peanut, almond, hazelnut, and β-lactoglobulin were prepared at 40 µg mL^{-1} in PBS. In contrast, the anti-gliadin, trypsin inhibitor, BSA, and anti-mouse were prepared at 80, 10, and 3 µg mL^{-1} in PBS. The BSA and anti-mouse antibody correspond to the negative and positive control assays. Fifty nanoliters of each solution were spotted with the non-contact printing device on the polycarbonate surface of the DVD in a microarray format (20 arrays per disk of 4 × 4 spots). Spots of 550 ± 38 µm in diameter (0.24 mm^2) were generated with a track pitch (center to center distance) of 1.0 mm. The capture bioreceptors were fixed on the polycarbonate surface by incubating the DVD at 37 °C for 16 h.

2.5. Multiplexed assay procedure

First, the DVD was washed with PBST, rinsed with distilled water, and thoroughly dried by handshaking. The calibration curve was performed by preparing serial dilutions (0 to 10000 ng mL^{-1} in extraction buffer PBST, 8% EtOH) of a cocktail solution that contained β-lactoglobulin, gliadin, trypsin inhibitor and the crude protein extracts of almond, peanut, and hazelnut. The assays for detecting peanut, almond, β-lactoglobulin, gliadin, and hazelnut followed a non-competitive assay format, while the soy assay followed a competitive assay format (Figure 1B). Each cocktail solution was doped with 1 µL of HRP-conjugated anti-soy antibody (0.2 µg mL^{-1} in PBS) to integrate both assay formats. 50 µL of the dilutions were dispensed on each microarray and incubated for 30 min. In this step, the soy allergens are recognized by the HRP-conjugated anti-soy antibody (competitive assay format) and the other allergens are captured by the immobilized antibodies (non-competitive assay format) **(Figure 2 A)**. Next, a washing step with PBST and distilled water was performed to remove the excess protein. Fifty microliters of a cocktail antibody solution were incubated on the microarrays for 30 minutes (generation of the immunosandwich in the non-competitive assays). This solution contained the HRP-conjugated antibodies for detecting peanut, almond, hazelnut, β-lactoglobulin, and gliadin at 2, 0.03, 2, 2 and 0.2 µg mL^{-1} in PBS, respectively. Finally, another washing was performed to remove the excess antibody, and a 10-minute incubation step with 50 µL of TMB solution was carried out. The immunoreaction was ended by rinsing the DVD surface with distilled water. The TMB precipitation upon the reaction with HRP generated a blue colorimetric signal on each microarray spot. The signal intensity depended on the concentration of allergen detected (directly and inversely proportional for the non-competitive and competitive assays, respectively), except on the spots related to the positive and negative controls **(Figure 2 B)**.

2.6. Assay quantification

The assay quantification was performed with a portable hacked disc drive and homemade software. The group has already described this analytical strategy (Morais et al., 2010). Briefly, a 650 nm laser beam interrogates the entire surface of the DVD, and an optoelectrical sensor detects the reflected beam. It correlates the variations in the optical power of the reflected beam with the concentration of specific allergens, which is proportional to the optical density of the TMB precipitate (Dobosz et al., 2022). Subsequently, the reflected signal is obtained from the quadrant photodiode of the optical pick-up unit (RF differential signal) and digitized using the data acquisition board **(Figure 2 C)**. The latter is processed by the homemade software (Biodisk), which provides an analytical signal upon subtracting the local background signal from the median signal value of the spot.

2.7. Food sample preparation

Gluten-free, egg-free and nut-free biscuits; gluten-free samples of eel and crab substitutes and allergen-free probiotic samples were kindly provided by Gullón

(Palencia, Spain), Angulas Aguinaga (Irura, Spain) and ADM Biopolis (Paterna, Spain). The protein extraction procedure started with the sample's crushing and homogenization with a hand blender at 10.000 min^{-1} for 5 min. 250 mg of the sample were mixed with 2 mL of the extraction buffer (PBST, 40% ethanol v/v, EtOH) at 950 rpm and at room temperature for 15 minutes. The protein extract was recovered by centrifugation at 3600 rpm for 15 minutes and diluted 1/5 (v/v) in PBST before the analysis.

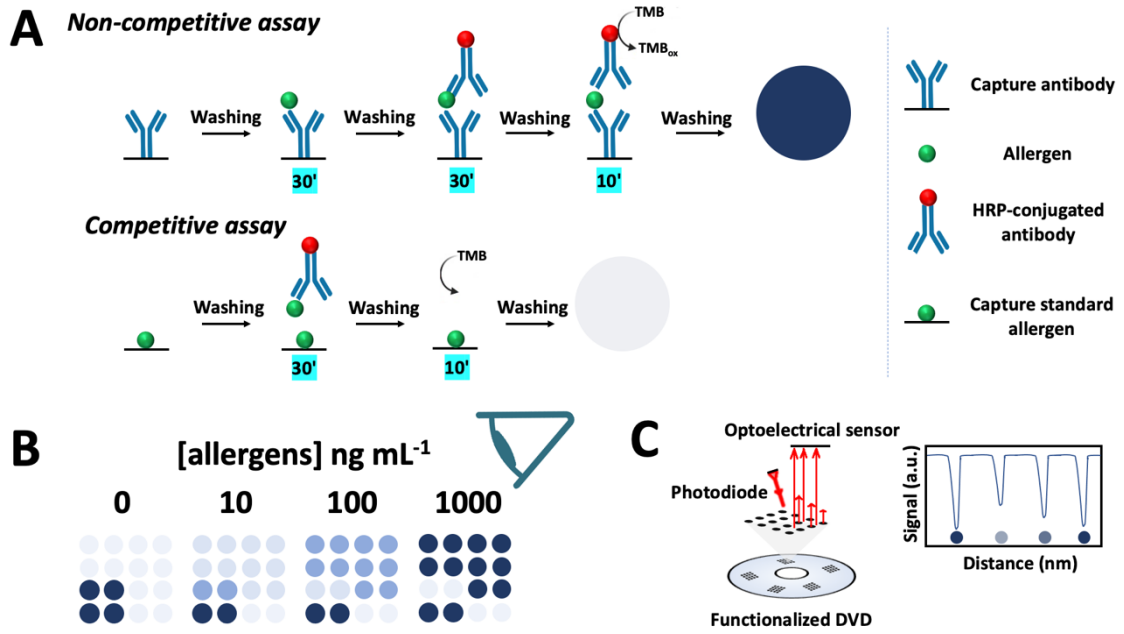

Figure 2. Schematic representation of the (A) Immunoassay procedure for the non-competitive and competitive assay formats. (B) Qualitative evaluation by the naked eye of a calibration curve for the simultaneous detection of 6 allergens. (C) Quantitative assessment of the assay using the homemade optoelectrical device.

3. Results

3.1. Development of individual assays

Individual assays were performed and optimized before moving towards the multiplexed detection of the allergens. The appropriate concentration of capture and detector bioreagents was achieved by searching for the conditions that enabled (1) the absence of non-specific signal in blank samples, where there is no allergen present, (2) the generation of the highest possible signals in positive assays and (3) linear dynamic ranges that covered at least two orders of magnitude (data not shown). Given that the raised almond, peanut, and hazelnut Mabs were produced using the crude protein extracts as the immunogens, these antibodies were expected to show reactivity against all proteins in the extract, including the allergenic ones. To this end, calibration curves were performed using serial dilutions of pure proteins (β-lactoglobulin, gliadin, and soybean trypsin inhibitor) and crude protein extracts of almond hazelnut and peanut (0 to 10000 ng mL^{-1}) in PBST 8% EtOH. The extracts were previously analyzed by SDS-PAGE,

confirming the presence of the major allergenic proteins (Figure S1 and Table S1) (J. Costa et al., 2012; Joana Costa et al., 2016; Hefle et al., 1995). The process included ethanol at 8% (v/v) into the phosphate buffer (see section 2.7). The collected data for each calibration curve was fitted to a 4-parameter logistic (sigmoidal) equation **(Figure S2)**. The limit of detection (LoD) was calculated as Optical intensity (LoD) = blank + 3 σ blank (i.e., the corresponding value of the blank sample plus three times its standard deviation). In contrast, the limit of quantification (LoQ) was calculated as Optical intensity (LoQ) = blank + 10 σ blank(Armbruster & Pry, 2008). **Table S2** shows the sensitivity in terms of LoD, LoQ, IC50, curve slope, the linear dynamic range (LDR), and R-squared values of each assay. The sensitivity (low ng mL^{-1} range) and broad linear dynamic range (2-orders of magnitude) achieved in all the assays are more than appropriate for detecting food-borne allergens. It ensures that the final concentration of the allergens will still be within the quantifiable range after the dilution of the extracted sample.

3.2. Development and optimization of multiplexed assay

Concentrations of the bioreceptors were then optimized to integrate the assays within a single microarray for multiplexing purposes. Concentrations were selected that prevented the generation of non-specific signals between the assays and promoted similar analytical parameters to those obtained in the individual assays. Identical to the previous section, calibration curves were performed using serial dilutions (0 to 10000 ng mL^{-1} in PBST, 8% EtOH) of a master mix solution that contained the crude protein extracts of almond, peanut, and hazelnut, and the pure β-lactoglobulin, gliadin, trypsin inhibitor proteins. **Figure 3A** shows a picture of the biosensor's microarray after the simultaneous detection of 0, 10, 100, and 1000 ng mL^{-1} of the analytes (check the microarray layout in Figure 1B). The signal intensity of the assays (except for the soy one that follows a competitive format) increased exponentially upon detecting higher concentrations of food allergens, and signal variations related to the detection of a blank sample (no target analyte), and a low (close to the LoD), medium (close to the IC50), and high target analyte concentration (close to signal saturation)) were clearly distinguished by naked eye. This proves that the proposed biosensor can provide semi-quantitative assay data. Moreover, the signal intensity in the positive and negative control assay remained constant upon evaluating increasing concentrations of allergens **(Figure S3)**. This result proves that, as expected, these assays are analyte-independent and can be used as a reference for the qualitative analysis of the food samples.

The collected data were fitted to a 4-parameter logistic (sigmoidal) **(Figure S4)**. To facilitate the comparison between different assays, normalized data (min-max normalization) is presented (Akanbi et al., 2015) **(Figure 3B)**. It was observed that the assays gave different linear dynamic ranges and curve slopes according to the binding affinities of the selected bioreceptors with the target analytes. The achieved analytical

parameters **(Table S3)** are more than appropriate considering other published approaches **(Table S4)**.

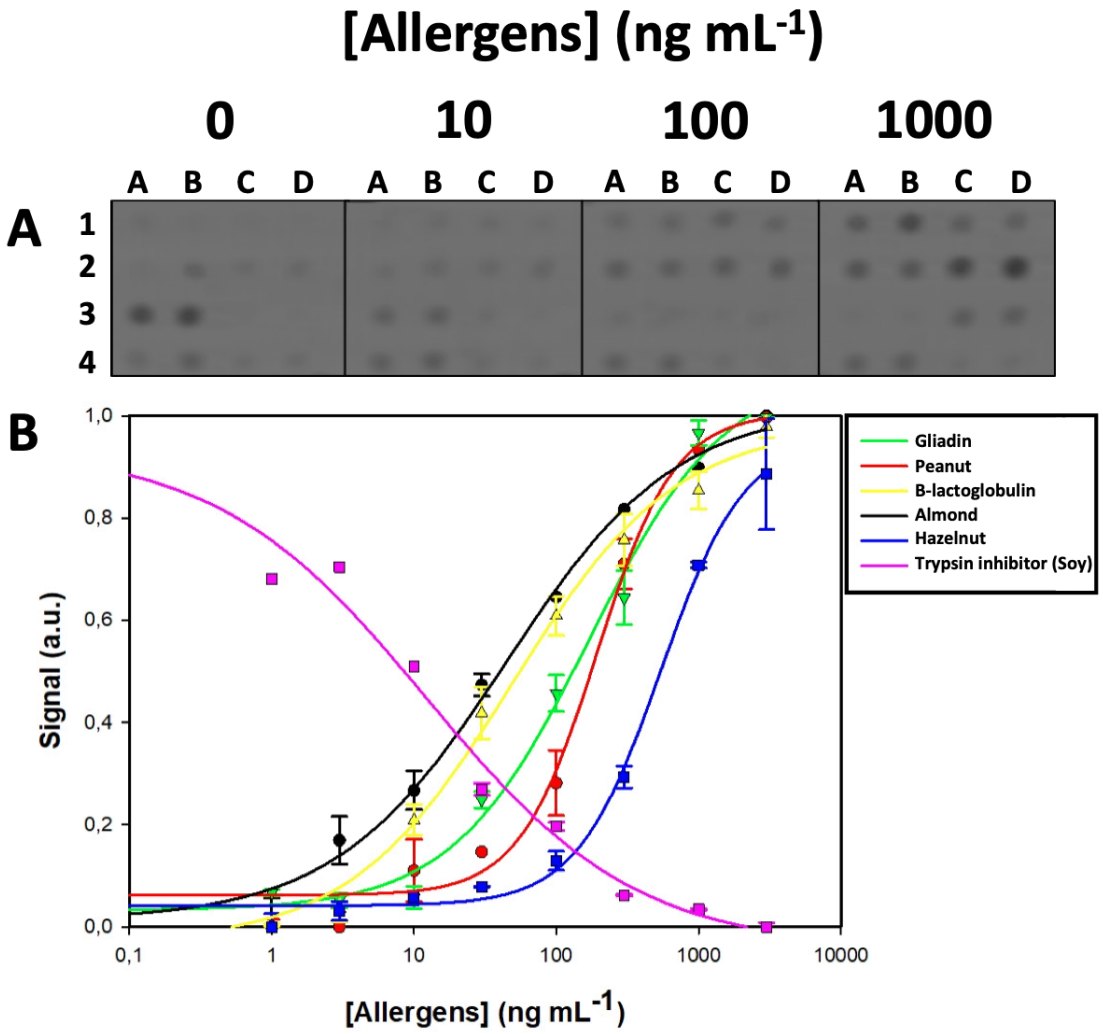

Figure 3. (A) Picture of the microarray after the simultaneous detection of serial dilutions of peanut, almond, β-lactoglobulin, gliadin, soy, and hazelnut allergens (0, 10, 100, 1000 ng mL^{-1} in PBST 8% EtOH) (Row 1 A,B: Peanut; Row 1 C,D: Almond; Row 2 A,B: β-lactoglobulin; Row 2 C,D: gliadin; Row 3 A,B: trypsin inhibitor (Soy); Row 3 C,D: Hazelnut; Row 4 A,B: Positive control; Row 4 C,D: Negative control). (B) Normalized calibration curves for the non-competitive (peanut, almond, β-lactoglobulin, gliadin, and hazelnut) and competitive (soy) immunoassays (three replicates per point).

3.3. Assay selectivity

The selectivity of the biosensor system was assessed by determining the cross-reactivity of the detector antibodies with the different capture antibodies and target allergens. Cross-reactivity must be checked in multiplexed systems to ensure that selected bioreagents are specific enough to provide an accurate positive signal. This information was achieved by measuring the signal generated in each microarray spot

after evaluating the IC_{50} concentration of each allergen. In this sense, signal generation should only appear in the spots related to the assay of interest, meaning that the target allergen is only recognized by its antibody pair. The cross-reactivity expressed in percentage was calculated by dividing the concentration of a particular allergen when detected with a non-intended matched pair by the known concentration of the allergen when seen with its intended matched pair (*Cross Reactivity Testing at Quansys Biosciences*, 2022). As observed in **Table 1**, all the bioreceptors show a cross-reactivity lower than 1% for the allergens, except for gliadin, which has a higher cross-reactivity with the antibodies raised against soy and hazelnut. The selectivity assay was then repeated without adding allergens (blank sample) to determine if the gliadin allergen caused the cross-reactivity or the anti-gliadin antibodies. Performing the assay with the blank sample, the signals for each assay were significantly different ($p = 0.0033$) than the one obtained in the negative control assay. As the soy assay is based on a competitive format, the highest signal was observed for the blank **(Figure S5)**. Therefore, the presence of gliadin gives a background/interferes with the soy and hazelnut capture bioreceptors. While aiming for more specific bioreceptors and assuming that the presented work is a proof-of-concept, the investigation continued with the analysis of the food samples, considering that part of the signal obtained in the hazelnut and soy spots could be related to the presence of gliadin in the sample.

Table 1. Cross-reactivity (%) of the antibodies when analyzing the IC_{50} concentration of the allergens (three replicates per assay).

Allergen	ALM	BLG	GLI	HAZ	PEA	SOY
ALM	-	0.01	0.41	0.01	0.01	0.01
BLG	0.02	-	1.08	0.02	0.02	0.02
GLI	0.04	0.27	-	0.04	0.04	0.04
HAZ	0.62	0.77	27.79	-	0.64	0.88
PEA	0.20	0.51	2.89	0.20	-	0.19
SOY	0.90	0.89	9.06	0.77	0.87	-

GLI: Gliadin; BLG: β-lactoglobulin; HAZ: Hazelnut; ALM: Almond; PEA: Peanut; SOY: Soya.

3.4. Analysis of food samples

The study evaluated the applicability of the multiplexed assay to confirm the declared allergen content in highly processed commercial food products. The food sample selection included a wide variety of food types children usually consume, including chocolate biscuits, seafood substitutes, and probiotics. Firstly, two different allergen-free biscuits were analyzed. Sample 1 declared no content of nuts, egg, gluten, and possible traces of milk allergens, while sample 2 claimed to be nut-free, egg-free, wheat-free, gliadin-free, and milk proteins-free. In addition, both samples were declared to contain soy lecithin. When evaluating these samples, signal intensities were primarily

obtained lower than those achieved in the negative control assay, except for the soy assay (**Figure S6)**. The high signal in the soy assay was not related to the detection of soy lecithin (known to have very little, if any, soy proteins (*Soy Allergy and Soy Lecithin*, 2019)) but rather to the nature of the competitive assay format (inversely proportional to the concentration of target analyte). Moreover, the signal intensities in the positive and negative control assays were similar to those obtained in the blank sample when evaluating the biscuits. This result proved that the complex matrix of the biscuits did not produce a matrix effect on the bioreceptors, and thus the assay enabled reliable results. Therefore, the qualitative evaluation of the signal intensities revealed that both biscuit samples lack food allergens. In quantitative terms, the concentration of gliadin, hazelnut, almond, and peanut in both biscuit samples was significantly lower than the limit of quantification of these assays **(see Table S3)**. In contrast, the concentration of soy and BLG allergens was negligible **(Table 2)**. Thus, the obtained results were consistent with the allergen content declared in the labeling of the biscuits.

The multiplexed solution also proved to be a good performance with seafood samples. The allergen content of 3 eel and 1 crab substitute food was evaluated. Eel sample 1 was declared to contain gluten, soy, and milk proteins, Eel sample 2 was declared to have gluten and soy but no milk proteins, Eel sample 3 was declared gluten-free but contained soy and milk proteins, and the crab sample declared no allergenic content. The obtained results were consistent with the labeling of these food products. In qualitative terms, a considerable decrease in the signal intensity in the soy assay was observed when evaluating the 3 eel substitute samples, which indicated the presence of soy allergens in these samples **(Figure S7)**. Moreover, the signal intensity in the gliadin assay was higher than in the blank and the positive control assay when evaluating the eel samples 1 and 2. The same was observed in the BLG assay when considering eel samples 1 and 3. Thereby, the presence of gliadin, BLG, and soy allergens in these samples could be qualitatively determined. These signal intensities were used to quantify the allergen content using the calibration curves shown in Figure 3 **(Table 2)**. The gliadin content in samples 1 and 2 was higher than the regulatory threshold (20 mg kg^{-1}). These food products were declared to have contained gliadin. There is no regulatory threshold in the case of BLG and soy, but these allergens were detected in the eel samples at quantities high enough to label the allergen (Madsen et al., 2020).

Finally, 4 probiotic samples (lyophilized bacteria), which declared no allergen content, were also analyzed. As observed in **Figure S8**, the signal intensities in almost all the assays were significantly similar ($p = 0.1668$) to those in the negative control assay and either equivalent or lower to those obtained when evaluating a blank sample ($p = 0.1539$). Therefore, the absence of allergens could be qualitatively determined in these samples. In quantitative terms, the levels of the detected allergens were either lower than the LoQ or almost 0 mg kg^{-1} in each of the four samples.

Table 2. Results of the simultaneous quantification of allergens in food samples (three replicates per sample).

SAMPLE	Detected quantity (mg kg^{-1})					
	ALM	BLG	GLI	HAZ	PEA	SOY
Biscuits-1	<LoQ	0.11 ± 0.01	<LoQ	<LoQ	<LoQ	<LoQ
Biscuits-2	<LoQ	0.10 ± 0.01	<LoQ	<LoQ	<LoQ	0.06 ± 0.01
Eel substitute-1	<LoQ	84.9 ± 6	385 ± 75	<LoQ	<LoQ	21.4 ± 0.8
Eel substitute-2	<LoQ	<LoQ	511 ± 25	<LoQ	<LoQ	16.5 ± 0.6
Eel substitute-3	<LoQ	82.2 ± 9	<LoQ	<LoQ	<LoQ	10.2 ± 0.3
Crab substitute	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.02 ± 0.01
Probiotic-1	<LoQ	0.13 ± 0.01	<LoQ	<LoQ	<LoQ	0.02 ± 0.01
Probiotic-2	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.07 ± 0.06
Probiotic-3	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.03 ± 0.02
Probiotic-4	<LoQ	0.10 ± 0.01	<LoQ	<LoQ	<LoQ	0.12 ± 0.02

GLI: Gliadin; BLG: β-lactoglobulin; HAZ: Hazelnut; ALM: Almond; PEA: Peanut; SOY: Soya. *LoQ: Limit of quantification

4. Conclusion

This is the first study in which the simultaneous quantification of traces of peanut, hazelnut, almond, β-lactoglobulin, gliadin, and soybean allergens in food products is performed on a multiplexed assay on a disc. The assay combines non-competitive and competitive immunoassay formats and includes positive and negative controls. The former provides versatility for detecting different allergens, while the latter offers assay reliability. Additionally, the miniaturization of the microarray enables the simultaneous analysis of 20 samples in 70 minutes, offering a higher efficiency than other multiplexed biosensing systems. The proposed solution has proved an appropriate sensitivity (LoD ranges from 0.1 to 95.4 ng mL^{-1} and LoQ ranges from 0.1 to 143.4 ng mL^{-1}) and robustness when evaluating food products with complex matrices. About the assay selectivity, almost all the bioreceptors have proved no cross-reactivity (<1%), except for the gliadin, which shows background/interferences with the hazelnut and soy antibodies. The multiplexed quantification method, as a proof of concept, with 10 processed commercial food products resulted in 100% accuracy when identifying the declared allergens. Thus, this approach presents promising applicability for the reliable and high-throughput screening of multiple allergens in commercial food products. Considering the global social and personal impact of food allergy worldwide, the availability of multiplexed systems that can provide results quickly and cost-effectively with a single assay has clear additional benefits for the food industry. Complete assay automation can be envisioned on advanced microfluidic platforms that could simplify the analytical protocol, including the sample treatment, and reduce the costs of expendable materials.

CRediT authorship contribution statement

A.S.: Conceptualization, Methodology, Investigation, Writing - Original Draft. **N.S.:** Investigation, Resources, Writing - Original Draft, Writing - Review & Editing. **D.B.:** Investigation. **C.A.:** Investigation. **Y.P.:** Investigation. **A.M.:** Supervision, Writing - Review & Editing, Funding acquisition. **S.M.:** Conceptualization, Methodology, Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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